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Phosphotyrosine in adherens junctions

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Th two mammalian mitochondrial stress proteins, grp 75 and hsp 58, transiently interact with newly synthesiz d mitochondrial proteins

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In mammalian cells, two of the so-called heat shock (hsp) or stress proteins are components of the mitochondria. One of these, hsp 58, is a member of the bacterial GroEL family, whereas the other, glucose-regulated protein (grp) 75, represents a member of the hsp 70 family of stress proteins. Owing to previous studies implicating a role for both the hsp 70 and GroEL families in facilitating protein maturation events, we used the method of native immunoprecipitation to examine whether hsp 58 and grp 75 might interact with other proteins of the mitochondria. In cells pulse-labeled with [^{35}S]-methionine, a significant number of newly synthesized mitochondrial proteins co-precipitated with either hsp 58 or grp 75. Such interactions appeared transient. For example, providing the pulse-labeled cells a subsequent chase period in the absence of radiolabel resulted in a reduction of co-precipitating proteins. If the pulse-chase labeling experiments were performed in the presence of an amino acid analogue, somewhat different results were obtained. Specifically, although many of the newly synthesized and analogue-containing proteins again were observed to co-precipitate with grp 75, the interactions did not appear transient, but instead were stable. Under steady-state labeling conditions, we also observed a portion of hsp 58 and grp 75 in an apparent complex with one another. On addition of ATP, the complex was dissociated. Accompanying this dissociation was the concomitant autophosphorylation of grp 75. On the basis of these observations, as well as previous studies examining the structure/function of the hsp 70 and GroEL proteins, we suspect that both hsp 58 and grp 75 interact with and facilitate the folding and assembly of proteins as they enter into the mitochondria.

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Introduction

Within the past few years, considerable progress has been realized concerning the structure and function of the so-called heat shock (hsp) or stress proteins. These proteins, most of which are expressed at modest or even high levels in the normal cell, exhibit increased expression in cells exposed to a variety of different stimuli. Most stimuli that increase the synthesis of the stress proteins represent various agents (e.g., metals or amino acid analogues) or treatments (e.g., elevated temperatures) that interfere with normal protein folding and assembly. Accordingly, recent data have shown that some of the stress proteins are integral in facilitating the proper folding, assembly, and posttranslational translocation of other cellular proteins.

Two of the stress proteins that are involved in protein maturation events are the so-called hsp 70 and GroEL proteins. In the case of hsp 70, there exist multiple and related forms of the protein, all of which bind ATP, but which are present within different cellular compartments. These include the cytosolic and nuclear hsp 72/73; the endoplasmic reticulum (ER) glucose-regulated protein (grp) 78 or BiP; and grp 75, which is present within mitochondria (for reviews see Lindquist, 1986; Craig 1985; Rothman, 1989; Welch, 1990). Additional forms of hsp 70 have recently been reported within chloroplasts and perhaps on the plasma membrane (Lakey *et al.*, 1987; Amir-Shapira *et al.*, 1990; Marshall *et al.*, 1990). All of the available evidence indicates that the various hsp 70 proteins bind to other proteins that are in the course of synthesis and/or posttranslational translocation and assembly. For example, the cytosolic hsp 70 proteins interact transiently with the nascent chains of polypeptides under synthesis on polysomes (Beckmann *et al.*, 1990). In addition, cytosolic hsp 70 appears to facilitate the translocation of proteins from the cytosol into the ER, mitochondria, and lysosome (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Chiang *et al.*, 1989; Sheffield, 1990). In the case of that form of hsp 70 present within the ER, grp 78 (or BiP) inter-

acts with polypeptides as they enter into the ER lumen (Haas and Wabl, 1983; Hendershot *et al.*, 1987; Kozutsumi *et al.*, 1988). In each case, the transient interaction of the target protein with the particular hsp 70 protein is thought to stabilize the maturing polypeptide until it assumes its final three-dimensional conformation. Once such a conformation is achieved, hsp 70 is released, presumably through ATP hydrolysis.

In a somewhat similar, but probably distinct, manner, the GroEL family of stress proteins also appears to be involved in protein maturation events. In bacteria, the GroEL protein has been shown to bind to and facilitate the assembly of lambda phage-head monomers into their final oligomeric prohead structure (Georgopoulos *et al.*, 1973; Sternberg, 1973; Rochan and Murialdo, 1983). In plants, a GroEL homologue has been identified and shown to be equivalent to the so-called Rubisco binding protein (Hemmingsen *et al.*, 1988). Here this GroEL-like protein mediates the proper assembly of the Rubisco small (S) and large (L) subunits into their final S_8L_8 oligomeric structure (Ellis and Hemmingsen, 1989). In yeast, mutations within the gene encoding the GroEL-like protein present in the mitochondria (referred to as hsp 60) result in the failure of a number of mitochondrial enzymes to correctly assume their final and active oligomeric structure (Chang *et al.*, 1989). Thus, both the hsp 70 and GroEL proteins appear essential for the proper folding and assembly of other cellular proteins.

In the present study we investigated whether the two mammalian stress proteins that are present in mitochondria, hsp 58 (a GroEL homologue) and grp 75 (a hsp 70 homologue), might also interact with other proteins. Using specific antibodies to each protein, we found that both grp 75 and hsp 58 interact with a number of newly synthesized proteins. Such interactions were both transient and sensitive to ATP. Finally, we observed that a portion of hsp 58 and grp 75 could be isolated in a stable complex with one another. Addition of ATP, however, resulted in a dissociation of the complex and an accompanying (auto) phosphorylation of grp 75. We discuss these results as they pertain to the possible mechanisms by which proteins are translocated into and assembled within the mitochondria.

Results

Previous studies have shown that two stress proteins, grp 75 and hsp 58, are present within mitochondria (McMullin and Hallberg, 1987; Craig *et al.*, 1989; Leustek *et al.*, 1989; Mizzen *et al.*, 1989). Like most nuclear-encoded mitochondrial proteins, hsp 58 and grp 75 are synthesized in the cytoplasm and then are post-translationally translocated into the organelle. Such import of the proteins into mitochondria can be prevented by incubating the cells with agents that cause depolarization of the mitochondria. For example, in cells treated with an uncoupler of oxidative phosphorylation, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), mitochondrial precursor proteins are observed to accumulate within the cytoplasm (Reid *et al.*, 1982). This is illustrated in Figure 1 in which HeLa cells were exposed to CCCP for 30 min and, while in the presence of the chemical, were labeled with [^{35}S]-methionine for 30 min (i.e., pulsed). After the labeling period, the radiolabel was removed and some of the cells were harvested. Alternatively, for some of the labeled cells, the labeling medium was removed and the cells further incubated in either the presence or absence of CCCP for an additional 30 min or 2 h (i.e., pulsed-chased). From both the pulsed and pulsed-chased cells, immunoprecipitations (under denaturing conditions) were performed using antibodies to hsp 58 or grp 75. As was shown previously, these antibodies appear specific for hsp 58 or grp 75 (Mizzen *et al.*, 1989). In addition, immunoprecipitation was performed using an antibody specific for the alpha subunit of the mitochondrial F_1ATPase.

In the cells labeled for 30 min in the presence of CCCP, appreciable amounts of the precursor forms of all three of these mitochondrial proteins were observed (Figure 1A). Removal of the radiolabel and further incubation of the cells in the presence of CCCP revealed the precursor form of hsp 58 to be relatively unstable. For example, after a 30-min chase period in the presence of CCCP, approximately one-half of the hsp 58 precursor had disappeared and by 2 h little or no hsp 58 precursor remained (Figure 1, B and C). In contrast, after a 2-h chase period, appreciable levels of the precursor forms of both the F_1ATPase α-subunit and grp 75 were still apparent (Figure 1, B and C). To examine the relative rate of conversion of the precursors into their mature forms, the cells, labeled in the presence of CCCP, were further incubated in the absence of CCCP for 30 min or 2 h. Conversion of hsp 58 to its mature form was complete by 30 min (Figure 1, D and E). Complete conversion of both the α-subunit and grp 75 precursors required significantly longer periods of time, but by 2 h both had been chased into their mature form (Figure 1F). At present, we do not understand the basis for either the

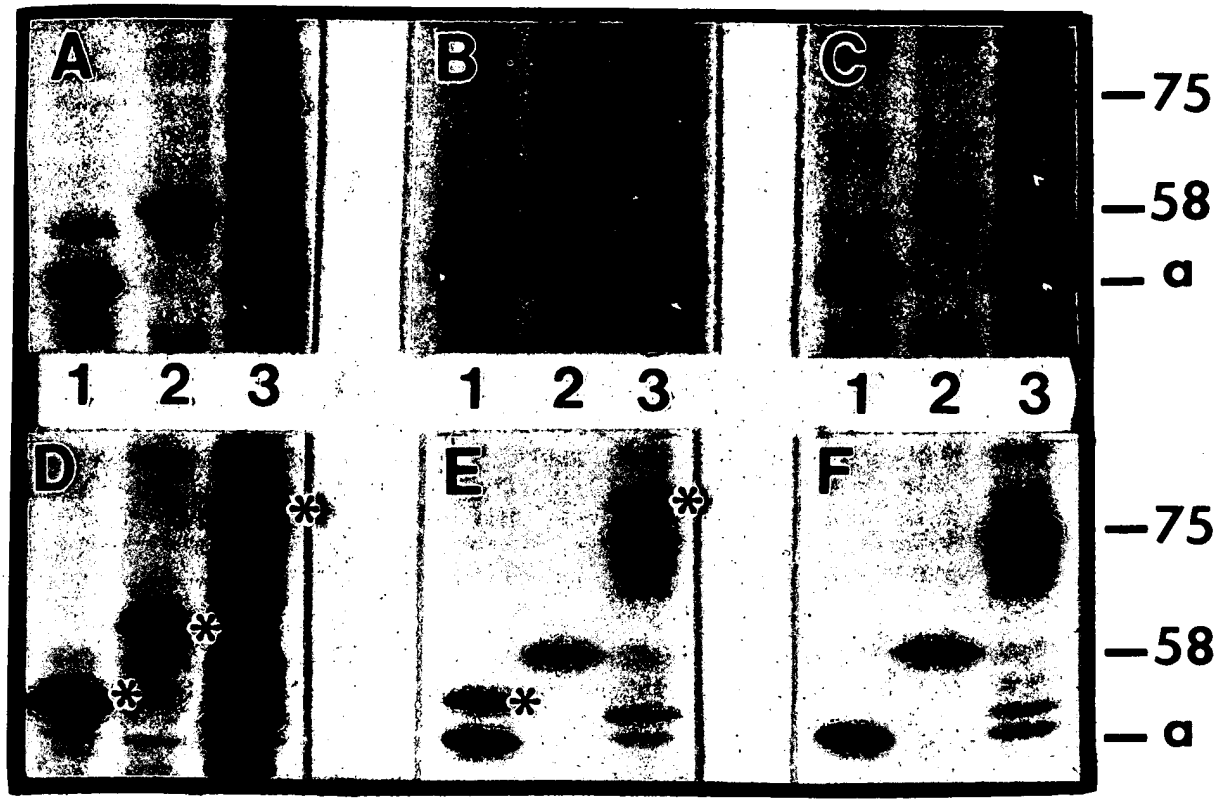

Figure 1. **The hsp 58 and grp 75 are synthesized as precursors and are posttranslationally translocated into the mitochondria with different kinetics.** HeLa cells, growing at 37°C, were treated with 20 μM CCCP, an uncoupler of oxidative phosphorylation, which prevents the uptake of mitochondrial precursors into the organelle. In the presence of the drug, the cells were labeled with [^{35}S]-methionine for 30 min. One plate of cells was immediately harvested with Laemmli sample buffer, whereas for the other plates, the radiolabel was removed and the cells further incubated, either in the presence or absence of CCCP, for an additional 30 min or 2 h. After the appropriate chase period, the cells were harvested in Laemmli sample buffer and incubated with ATP, and the relative amounts of the precursor and mature forms of the F_1ATPase α-subunit (lane 1), hsp 58 (lane 2), or grp 75 (lane 3) were determined by denaturing immunoprecipitation. (A–C) the relative amounts of the precursor forms of the three mitochondrial proteins that remain during the pulse-chase protocol in the presence of CCCP. (D–F) the relative rates of conversion of the precursors (*) into their mature form after removal of CCCP. (A) cells pulse-labeled for 30 min in the presence of CCCP; (B) as in A and then chased for 30 min in the presence of CCCP; (C) as in A and then chased for 2 h in the presence of CCCP. (D) cells pulse-labeled for 30 min in the presence of CCCP; (E) as in D and then chased for 30 min in the absence of CCCP; (F) as in D and then chased for 2 h in the absence of CCCP.

shorter half-life or the faster rate of conversion of hsp 58 compared with the two other mitochondrial proteins.

Owing to previous studies implicating a role for both the hsp 70 and GroEL (e.g., hsp 58) families of stress proteins in facilitating protein maturation events, we examined for possible interactions of both grp 75 and hsp 58 with other mitochondrial proteins. Our approach to this question was similar to that used to establish that the cytosolic hsp 72/73 interacts with the nascent chains of newly synthesized proteins (Beckmann *et al.,* 1990). Specifically, we examined possible protein–protein interactions by the method of immunoprecipitation under native conditions. Cells were steady-state labeled with [^{35}S]-methionine for 2 h, the label was removed, and the cells were further incubated for 1 h at 37°C. After hypotonic lysis of the cells, a crude mitochondrial pellet was prepared. Because both the cytosolic (e.g., hsp 72/73) and ER (e.g., grp 78 or BiP) forms of hsp 70 have been shown to exhibit ATP-sensitive interactions with other proteins (Haas and Wabl, 1983; Hendershot *et al.,* 1987; Kozutsumi *et al.,* 1988; Beckmann *et al.,* 1990), the crude mitochondrial pellet was solubilized in nonionic detergent and split in half. To one portion we added the ATP-depleting enzyme apyrase, whereas to the other one-half we added 2 mM ATP. After incubation at 4°C for 15 min, the two mitochondrial preparations were clarified and native immunoprecipitation analysis was performed using antibodies specific for hsp 58, grp 75, and the F_1ATPase alpha subunit (Figure 2A). In those mitochondrial extracts first depleted of ATP, the α-subunit was

immunoprecipitated with only a few minor co-precipitating proteins. Under these conditions, (i.e., 4°C and detergent) the intact F_1ATPase is relatively unstable and, therefore, the antibody precipitates only the free α-subunit (L. Krakowski, personal communication). Immunoprecipitation with the hsp 58 antibodies resulted in the isolation of hsp 58 along with a second prominent protein of ~70 kDa, which migrated identically to that of grp 75. In addition, a few other minor proteins were observed to co-precipitate with hsp 58 in the ATP-depleted lysates. The anti-grp 75 antibody precipitated grp 75 and a few other proteins in the 40-kDa range, as well as some higher molecular mass proteins. In those mitochondrial extracts first incubated with ATP, again the α-subunit was observed to precipitate with only a few, minor associated proteins. In the case of the hsp 58 immunoprecipitates, however, little or none of the aforementioned co-precipitating 70-kDa protein was observed in the lysates first treated with ATP. Similarly, in the presence of ATP, there appeared to be fewer proteins co-precipitating with grp 75.

A similar analysis from cells placed under stress provided somewhat different results. For these studies, cells were stressed by exposure to an analogue of proline (L-azetidine-2-carboxylic acid, Azc) for 4 h and then labeled for 2 h with [^{35}S]-methionine in the presence of Azc. The radiolabel was removed, and the cells were further incubated for 1 h while still in the presence of Azc. Crude mitochondria were prepared, solubilized in detergent, split into two equal portions, and then either treated with apyrase (to deplete ATP) or incubated with 2 mM ATP. Native immunoprecipitations employing antibodies to the three mitochondrial proteins were performed and analyzed as de-

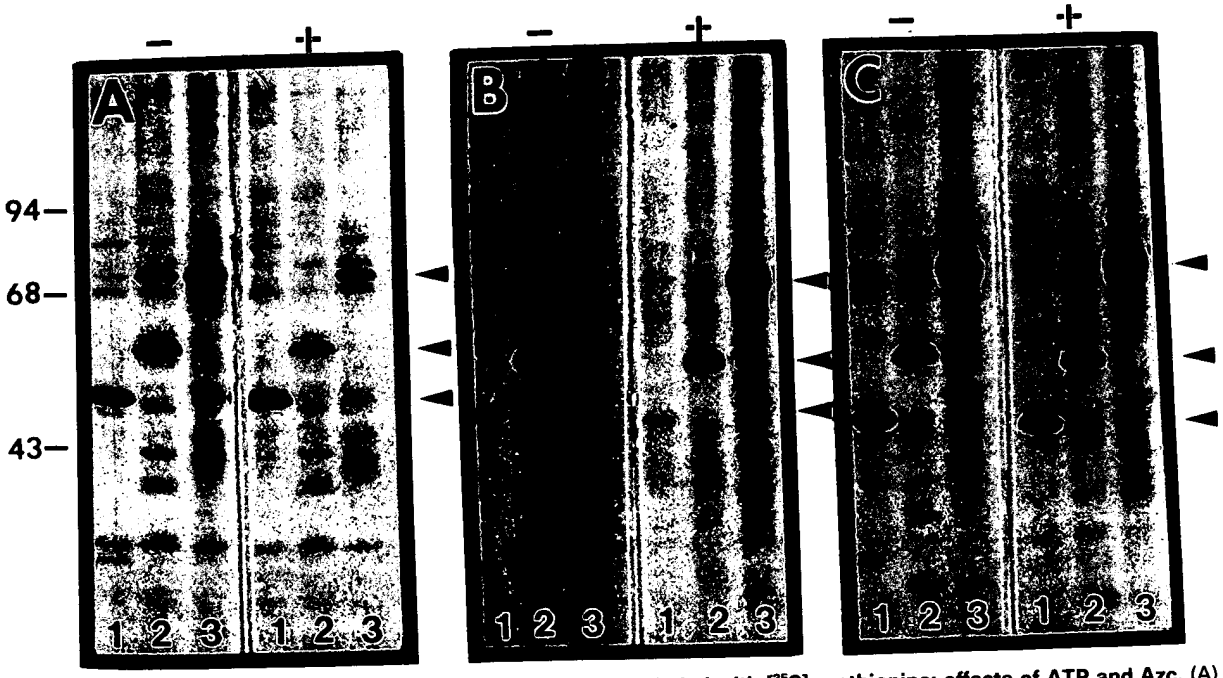

Figure 2. **Analysis of protein complexes in cells steady-state labeled with [^{35}S]-methionine: effects of ATP and Azc.** (A) HeLa cells, growing on plastic dishes at 37°C, were labeled for 2 h with [^{35}S]-methionine, the label was removed, and the cells were further incubated in the absence of label for 1 h. Crude mitochondria were prepared; to one-half of the mitochondrial lysate was added the ATP-depleting enzyme apyrase, whereas to the other one-half was added 2 mM ATP. After a 15-minute incubation at 4°C, the lysates were clarified and native immunoprecipitations were performed using antibodies against the α-subunit of the F_1ATPase (lane 1), hsp 58 (lane 2), and grp 75 (lane 3). Lysates depleted of ATP via apyrase (−) and lysates to which ATP was added (+) are indicated at the top of each panel. (B) Similar to the experiment shown in A, HeLa cells were treated with the proline analogue (Azc, 5 mM) for 4 h and labeled with [^{35}S]-methionine for 2 h in the presence of the analogue, the radiolabel was removed, and the cells were further incubated in the presence of Azc for 1 h. The cells were harvested and a crude mitochondrial lysate was prepared. To one-half of the lysate was added apyrase whereas to the other one-half was added 2 mM ATP. Immunoprecipitations were then performed as described in A. (C) Cells were treated with Azc for 4 h, the analogue was removed, and the cells were incubated in the absence of Azc for 1 h. The cells were then labeled with [^{35}S]-methionine for 2 h, the label was removed, and the cells were further incubated for 1 h. Crude mitochondria were prepared; to one-half was added apyrase whereas to the other was added 2 mM ATP and immunoprecipitations performed as described in A. The positions of grp 75, hsp 58, and the F_1ATPase α-subunit are shown in descending order on the right and the positions of molecular mass markers are indicated on the left.

scribed above (Figure 2B). In the case of the α-subunit, relatively little of the radiolabeled protein was precipitated in either the ATP-depleted or -supplemented lysates. This is likely due to its decreased synthesis (owing to the cells now being in a stress response), as well as its shorter half-life because of the incorporated amino acid analogue. Both of the stress proteins, hsp 58 and grp 75, were synthesized at high levels in the cells placed under stress via exposure to Azc. Now, however, significantly less of the radiolabeled and analogue-containing 70-kDa protein was observed to co-precipitate with the hsp 58 antibody in the Azc-treated lysates first depleted of ATP. Addition of ATP to the lysate before the immunoprecipitation resulted in even less of the co-precipitating 70-kDa protein. In the case of grp 75, a significant number of polypeptides were now observed to co-precipitate in the Azc-treated and ATP-depleted lysates. One of these co-precipitating proteins migrated identically with that of hsp 58. Somewhat fewer proteins co-precipitated with grp 75 in the Azc-treated cells first incubated with ATP. These differences in co-precipitating proteins observed in the Azc-treated cells were fully reversible. For example, removal of the analogue and subsequent [^{35}S]-methionine labeling of the cells under normal conditions resulted in a pattern of co-precipitating proteins identical to that observed for the unstressed cells (e.g., compare Figure 2, C with A).

To more clearly distinguish and possibly identify some of the proteins co-precipitating with either hsp 58 or grp 75, we analyzed some of the immunoprecipitates shown in Figure 2 by two-dimensional gel electrophoresis. Such analysis revealed that the 70-kDa protein observed to co-precipitate with the hsp 58 antibody in the ATP-depleted normal cell lysates was in fact grp 75 (Figure 3A). In cells treated with Azc and then labeled in the presence of Azc, radiolabeled grp 75 was no longer present in the hsp 58 immunoprecipitate (Figure 3B). Conversely, under normal conditions, little or no hsp 58 was observed to co-precipitate with grp 75 (Figure 3C). In contrast, hsp 58 synthesized in the presence of Azc was now found in the grp 75 immunoprecipitates (Figure 3D).

Although somewhat complicated, our data to this point had shown that the two mitochondrial stress proteins hsp 58 and grp 75 existed, in part, in a complex with one another. Specifically, in normal cells antibodies against hsp 58 precipitated both hsp 58 and grp 75, but only from those mitochondrial lysates first depleted of ATP. In the cells labeled in the presence of Azc, however, the radiolabeled and analogue-containing grp 75 did not co-precipitate with the hsp 58 antibody. Conversely, under normal conditions, antibodies to grp 75 did not co-precipitate hsp 58. If, however, the cells were labeled in the presence of Azc, the radiolabeled and analogue-containing hsp 58 now was observed to co-precipitate with the grp 75 antibody, and again the interaction appeared sensitive to ATP.

To determine whether hsp 58 or grp 75 might exhibit transient interactions with other newly synthesized mitochondrial proteins, we performed these same sorts of experiments using pulse and pulse-chase labeling techniques. Cells were pulse-labeled at 37°C for 15 min with [^{35}S]-methionine, immediately harvested by lysis in nonionic detergents, and depleted of ATP via incubation with apyrase (pulsed). For a second plate of the pulse-labeled cells, the medium was removed and the cells were further incubated at 37°C in the absence of radiolabel for 1 h, then harvested and treated with apyrase (pulsed-chased). In the pulse-labeled cells a significant number of polypeptides were observed to co-precipitate with either the hsp 58 or grp 75 antibodies (Figure 4A, lanes 3, 4 in P). If, however, the pulse-labeled cells were provided a subsequent 1-h chase period in the absence of radiolabel, significantly fewer co-precipitating proteins were observed (Figure 4A, lanes 3, 4 in C). To ensure that the proteins co-precipitating with grp 75 and hsp 58 were indeed newly synthesized mitochondrial proteins, these pulse-chase experiments were repeated, but this time an enriched fraction of mitochondria was isolated, treated with the nonionic detergent and apyrase, and then examined by native immunoprecipitation. As before, a large number of newly synthesized mitochondrial proteins were observed to co-precipitate with either the hsp 58 or grp 75 antibodies from the mitochondria isolated from the pulse-labeled cells (Figure 4B, lanes 3, 4 in P). After the 1-h chase period in the absence of radiolabel, again significantly fewer proteins were observed to co-precipitate (Figure 4B, lanes 3, 4 in C).

These same pulse-chase experiments were repeated in cells placed under stress by exposure to the amino acid analogue Azc. Specifically, cells were treated with Azc for 4 h, and, while in the presence of the analogue, the cells were labeled for 15 min with [^{35}S]-methionine. One plate of cells was harvested immediately (pulsed), whereas for a second plate, the radiolabel was removed and the cells were further incubated in the presence of Azc for 1 h (pulsed-chased). The cells were harvested; and—from both the pulsed and pulsed-chased cells—

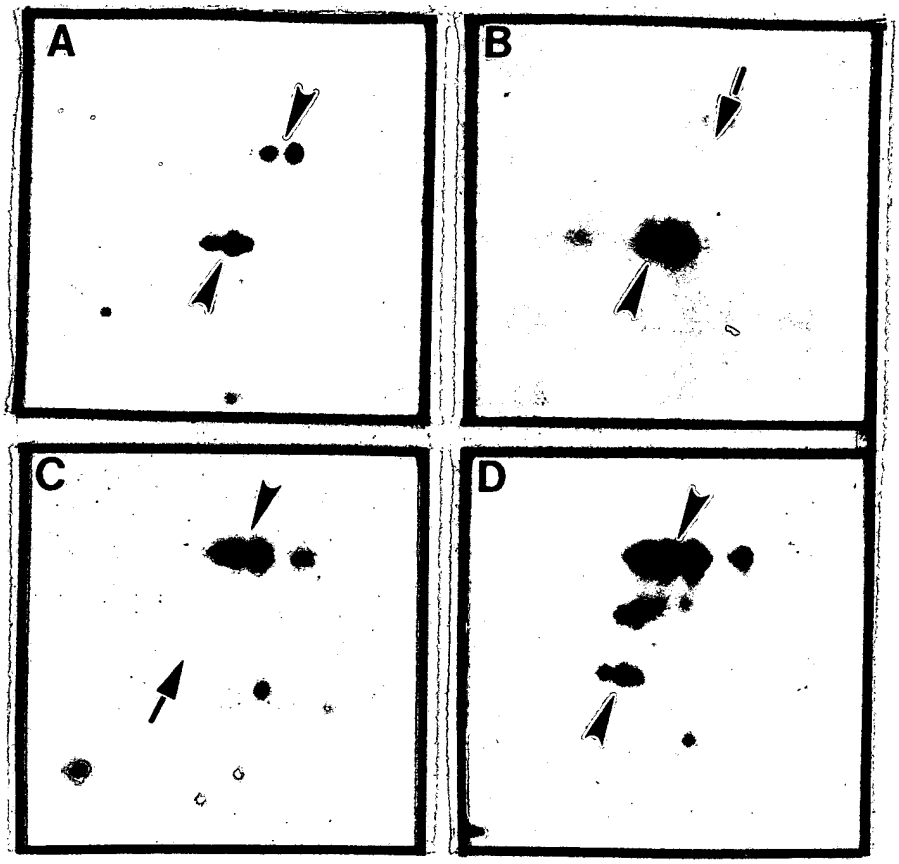

Figure 3. **Two-dimensional gel analysis of hsp 58 and grp 75 immunoprecipitates from steady-state-labeled cells.** Relevant immunoprecipitation products from Figure 2 were analyzed by two-dimensional gel electrophoresis. Shown are only the hsp 58 and grp 75 immunoprecipitates from cells first treated with apyrase to deplete ATP. Indicated in each panel are the positions of grp 75 (downward-pointing arrowhead) and hsp 58 (upward-pointing arrowhead). (A) anti-hsp 58 immunoprecipitate from control cells. (B) anti-hsp 58 immunoprecipitate from cells labeled in the presence of Azc. (C) anti-grp 75 immunoprecipitate from control cells. (D) anti-grp 75 immunoprecipitate from cells labeled in the presence of Azc. Shown are only those regions of the gel containing grp 75 and hsp 58. The downward and upward pointing arrows in B and C indicate where grp 75 and hsp 58, respectively, would migrate if present in the immunoprecipitate.

crude mitochondria were prepared, lysed with nonionic detergent, and treated with apyrase. Although synthesis of most cellular proteins, with the exception of the stress proteins, was decreased in the Azc-treated cells, we again observed a significant number of the newly synthesized and analogue-containing proteins to co-precipitate with grp 75 (Figure 4C, lane 4 in P). In the case of hsp 58, some newly synthesized proteins co-precipitated, but significantly the number was fewer than observed in the normal, unstressed cells (Figure 4C, lane 3 in P). In the case of the cells pulse-labeled and chased in the presence of Azc, most of the newly synthesized and analogue-containing proteins were still observed to co-precipitate with grp 75 (Figure 4C, lane 4 in C). Similarly, a number of proteins also appeared still to co-precipitate with hsp 58 (Figure 4C, lane 3 in C).

In summary, the pulse-chase experiments demonstrated that, under normal labeling conditions, a large number of newly synthesized proteins were interacting transiently with either grp 75 or hsp 58. Specifically, in the pulse-labeled cells a number of newly synthesized proteins co-precipitated with either hsp 58 or grp 75. After a 1-h chase period in the absence of the radiolabel, significantly fewer proteins were observed to co-precipitate. In the case of the pulse-chase studies performed in the presence of Azc, again, many newly synthesized proteins were observed to co-precipitate with grp 75. However, in contrast to the situation with the unstressed cells, many of the newly synthesized and analogue-containing proteins now remained in a stable complex with grp 75 after the 1-h chase period.

Both the hsp 58 and grp 75 immunoprecipitates from cells pulsed and pulsed-chased under normal conditions were analyzed by two-dimensional gels. In both the pulsed and pulsed-chased cells, the hsp 58 antibodies precipitated both hsp 58 and grp 75 (Figure 5, A and B). Apparently, the interaction between these two proteins occurs rapidly and is relatively stable in the absence of ATP. We also noted the presence of an ~50-kDa protein co-precipitating with hsp 58 in only the pulse-labeled cells and not after the 1-h chase period. This protein represents the β-subunit of the F_1ATPase as determined by its size and isoelectric point (indicated by arrows in Figure 5, A and B) (Mizzen

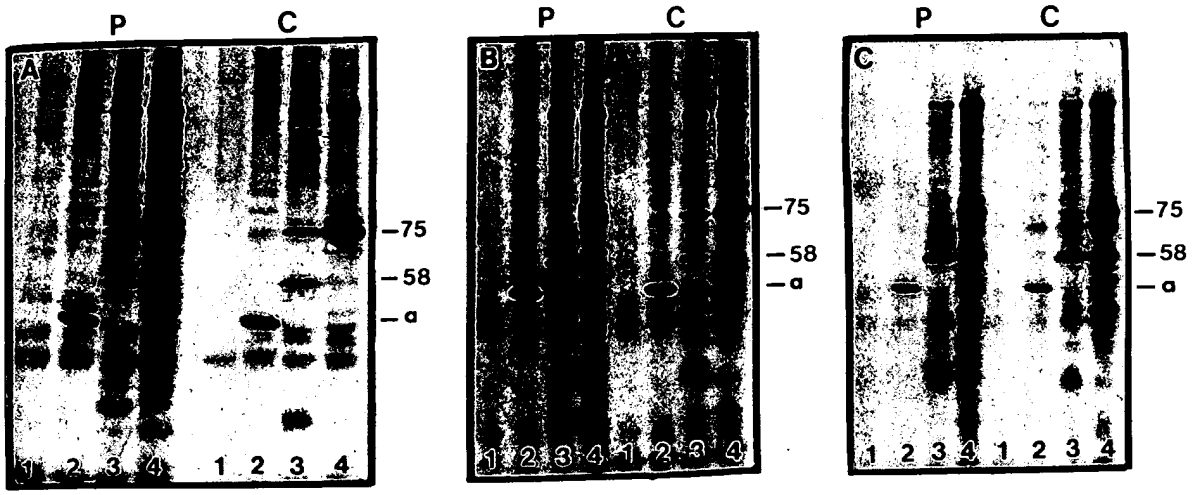

Figure 4. **HSP 58 and grp 75 exhibit transient interactions with newly synthesized proteins.** HeLa cells, growing at 37°C, were incubated in the absence or presence of 5 mM Azc for 4 h. The cells were then pulse-labeled with [^{35}S]-methionine in either the absence or presence of Azc for 15 min. Some of the cells were immediately harvested; cell lysates were prepared and treated with apyrase. Alternatively, the pulse-labeled cells were lysed by incubation in hypotonic buffer and a crude mitochondrial pellet was prepared, then treated with apyrase. For the other pulse-labeled cells (done either in the presence or absence of Azc), the radiolabel was removed and the cells were further incubated in either the presence or absence of Azc for 1 h. Again, whole cell lysates or, alternatively, a crude mitochondrial pellet was prepared and treated with apyrase to deplete ATP. Native immunoprecipitations were then performed using control rabbit anti-mouse IgG antibodies (lane 1), rabbit anti-F_1ATPase α-subunit (lane 2), rabbit anti-hsp 58 (lane 3), or rabbit anti-grp 75 (lane 4). (A) Immunoprecipitates from whole cell lysates from cells pulse-labeled for 15 min (P) or pulse-labeled for 15 min and chased for 1 h (C). (B) Immunoprecipitates from crude mitochondria isolated from cells pulse-labeled for 15 min (P) or pulse-labeled for 15 min and chased for 1 h (C). (C) Immunoprecipitates from crude mitochondria isolated from cells treated with Azc and pulse-labeled for 15 min in the presence of Azc (P) or pulse-labeled for 15 min and chased in the presence of Azc for 1 h (C). The positions of grp 75, hsp 58, and the F_1ATPase α-subunit are indicated in descending order on the right of each panel.

et al., 1989). In the case of the grp 75 immunoprecipitates, a considerable number of newly synthesized proteins were observed to co-precipitate in the pulse-labeled cells (Figure 5C). One of these was hsp 58; another was the aforementioned F_1ATPase β-subunit. After the 1-h chase period, significantly less of either the hsp 58 or the β-subunit were observed within the grp 75 immunoprecipitate (Figure 5D).

Having established that a portion of hsp 58 and grp 75 interact with one another, but only in those lysates first depleted of ATP, we wanted to investigate how ATP might be regulating their interaction. Previous studies had demonstrated that grp 75, like the other members of the hsp 70 family, is an ATP-binding protein (Mizzen *et al.,* 1989; Welch and Feramisco, 1985). In addition, others had shown that the prokaryotic homologue of grp 75, the so-called dnaK protein, displayed autophosphorylation activities as a function of low pH (e.g., 6.0) and added calcium (Zylicz *et al.,* 1983; Leustek *et al.,* 1989). Therefore, mitochondria were purified from guinea pig livers. From the isolated mitochondria we also purified grp 75. Using both the isolated mitochondria and purified grp 75, we examined the possible kinase activity of grp 75 as a function of low or high pH and added cations such as Ca^{2+} or Mg^{2+}. Only under conditions of low pH (e.g., pH 6.0) and added calcium did we observe significant phosphorylation of grp 75, either in the whole mitochondria or with the purified protein (Figure 6).

Because the complex between hsp 58 and grp 75 was observed only in those lysates first depleted of ATP, we examined whether release of grp 75 from the hsp 58 immunoprecipitates would occur as a function of added ATP and whether release might be accompanied by grp 75 autophosphorylation. Cells were steady-state labeled with [^{35}S]-methionine and cell lysates were prepared and immediately depleted of ATP. Native immunoprecipitations with the hsp 58 antibody were performed to isolate the hsp 58-grp 75 complex. The immune complex, still present on the protein A–sepharose beads, was incubated under the optimal conditions for grp 75 autophosphorylation (i.e., pH 6.0 and added calcium). To the complex was added either 1 μM ATP or 10 μM of the nonhydrolyzable ATP analogue, AMPPNP, and the samples were incubated at 30°C for 30 min. After the incubation, the mixture was centrifuged, the supernatant was removed, and the pellet containing the protein A-antibody-antigen complex was

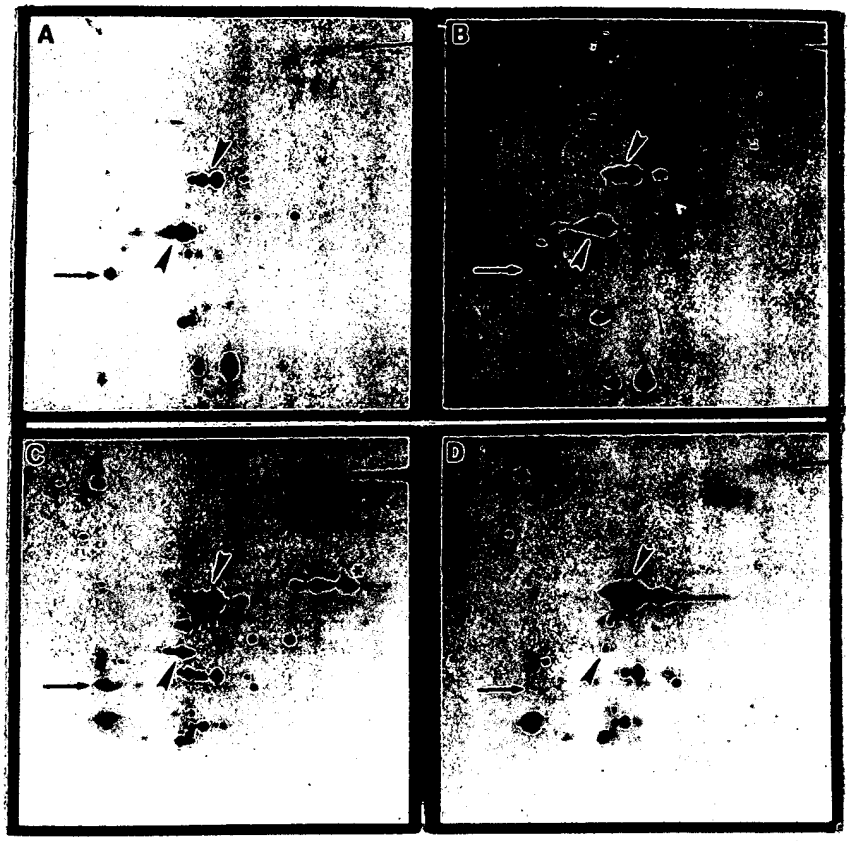

Figure 5. **Analysis of hsp 58 and grp 75 immunoprecipitates from pulse-labeled and pulse-labeled and chased cells by two-dimensional gels.** To more clearly resolve the proteins present within some of the immunoprecipitates shown in Figure 4, the samples were analyzed by two-dimensional gel electrophoresis. The downward-pointing arrowhead indicates grp 75; the upward-pointing arrowhead indicates hsp 58; and the left-to-right arrow indicates the position of the β subunit of the F_1 ATPase. The * in C represents the precursor form of grp 75. (A) hsp 58 immunoprecipitate from pulse-labeled cells. (B) hsp 58 immunoprecipitate from pulse-labeled and chased cells. (C) grp 75 immunoprecipitate from pulse-labeled cells. (D) grp 75 immunoprecipitate from pulse-labeled and chased cells.

washed with phosphate-buffered saline (PBS). Proteins present within either the supernatant or pellet were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; an equal percentage of both the supernatant and pellet were analyzed). Incubation of the immune complex with the ATP analogue AMPPNP resulted in the release of only a small amount of grp 75 into the supernatant. Incubation of the immune complex with 1 μM ATP, however, resulted in the release of significant amounts of grp 75 (Figure 7A). No other proteins present within the hsp 58 immune complexes were significantly released after the incubation with ATP. These experiments using the [^{35}S]-methionine-labeled cells were repeated, but this time the incubations were performed in the presence of ATP or the ATP analogue supplemented with γ-[^{32}P]-ATP. In the case of added ATP plus γ-[^{32}P]-ATP, again, grp 75 was released into the supernatant and, as determined by liquid scintillation counting, contained incorporated [^{32}P] (Figure 7B lanes 3, 4). Incubation of the immune complex with the nonhydrolyzable ATP analogue plus γ-[^{32}P]-ATP resulted in only a slight release of grp 75 that contained 10-fold less [^{32}P] radioactivity (Figure 7B lanes 1, 2). As a final step in this analysis, native immunoprecipitations from unlabeled and ATP-depleted cell lysates using the grp 75 antibody were performed. The immune complexes were incubated with 1 μM ATP supplemented with 10 μCi of γ-[^{32}P]-ATP and either the material released into the supernatant or that which remained bound in the immune complex was analyzed. Somewhat to our surprise, a portion of grp 75 was released into the supernatant and contained radiolabeled phosphate (Figure 7C). In addition, a portion of grp 75 that remained bound to the antibody protein A–sepharose also exhibited autophosphorylation.

Discussion

Within the past few years it has become evident that the proper folding and assembly of newly synthesized proteins require the participation of accessory molecules, now being referred to as molecular chaperones (Hemmingsen *et al.*, 1988; Ellis and Hemmingsen, 1989; Rothman, 1989). Although folding of the polypeptide is still believed to be dictated by its primary sequence, molecular chaperones are thought to facilitate the process by reducing nonproductive or improper folding pathways, thereby maintaining both high fidelity and kinetics of protein folding

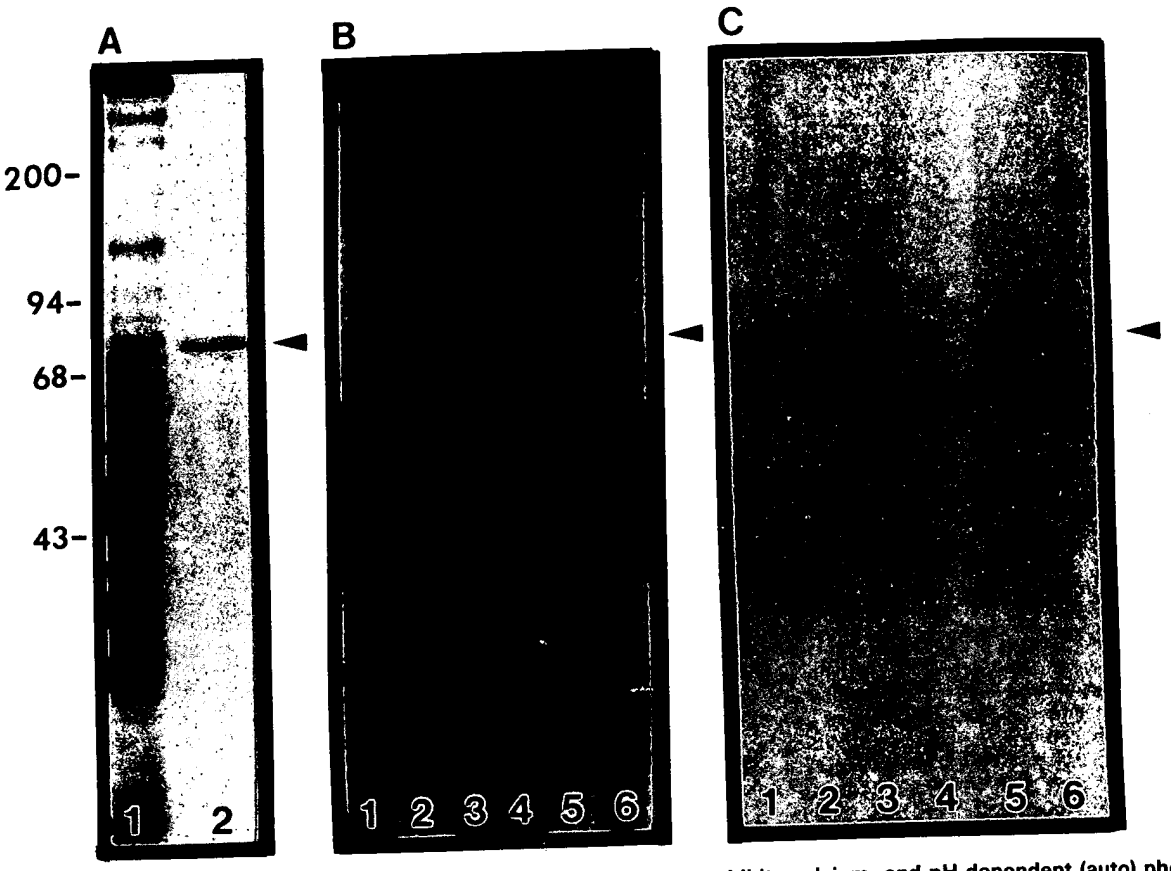

Figure 6. **Purified grp 75 or grp 75 present in isolated mitochondria exhibits calcium- and pH-dependent (auto) phosphorylation.** Mitochondria were purified from guinea pig livers as described in Materials and methods. In addition, grp 75 was purified from the isolated mitochondria as described previously (Mizzen *et al.*, 1989). Both the mitochondria and purified grp 75 were examined by SDS-PAGE and the proteins present were stained with Coomassie blue. (A) the Coomassie blue–stained proteins present in the isolated mitochondria (lane 1) or the purified grp 75 (lane 2). The isolated mitochondria or purified grp 75 were incubated at either pH 6.2 or pH 8.0 and supplemented with either $CaCl_2$ or $MgCl_2$ and then incubated with 1 μM unlabeled ATP plus 10 μCi of γ-[^{32}P]-ATP at 30° for 30 min. After the incubation, Laemmli sample buffer was added, the samples were incubated at 100°C for 5 min, and the radiolabeled proteins were examined by SDS-PAGE and autoradiography. (B) the mitochondrial extracts; (C) the purified grp 75. (Lane 1) incubation at pH 6.2, in the presence of 10 mM EDTA/EGTA; (lane 2) incubation at pH 6.2 in the presence of 10 mM $CaCl_2$; (lane 3) incubation at pH 6.2 in the presence of 10 mM $MgCl_2$; (lane 4) incubation at pH 8.0 in the presence of 10 mM EDTA/EGTA; (lane 5) incubation at pH 8.0 in the presence of 10 mM $CaCl_2$; (lane 6) incubation at pH 8.0 in the presence of 10 mM $MgCl_2$. Indicated to the right of each panel is the position of grp 75; molecular mass markers are indicated on the left.

in the cell. A number of these molecular chaperones have now been identified, many of which represent members of the so-called heat shock or stress protein family. The stress proteins, most of which are constitutively produced, exhibit increased expression in cells exposed to a number of agents or treatments. Most of these agents/treatments interfere with normal protein-folding pathways. Consequently, increased expression of the stress proteins is thought to provide the cell under stress a means by which to handle the increasing problems occurring with respect to proper protein folding and assembly events.

Because some of the other members of the hsp 70 family (e.g., cytosolic hsp 72/73 and ER grp 78 or BiP) as well as the GroEL proteins appear to function like molecular chaperones, we investigated whether the mitochondrial grp 75 or hsp 58 might similarly interact with proteins that are in the course of maturation. Using pulse-chase labeling techniques and native immunoprecipitation analysis, we observed both hsp 58 and grp 75 to exhibit transient interactions with a significant number of newly synthesized mitochondrial proteins. Such interactions were only observed if ATP levels were first depleted before the immunoprecipitation analysis. Two of the newly synthesized mitochondrial proteins co-precipitating with grp 75 were hsp 58 and the β-subunit of the F_1ATPase. These two proteins, as well as a number of other

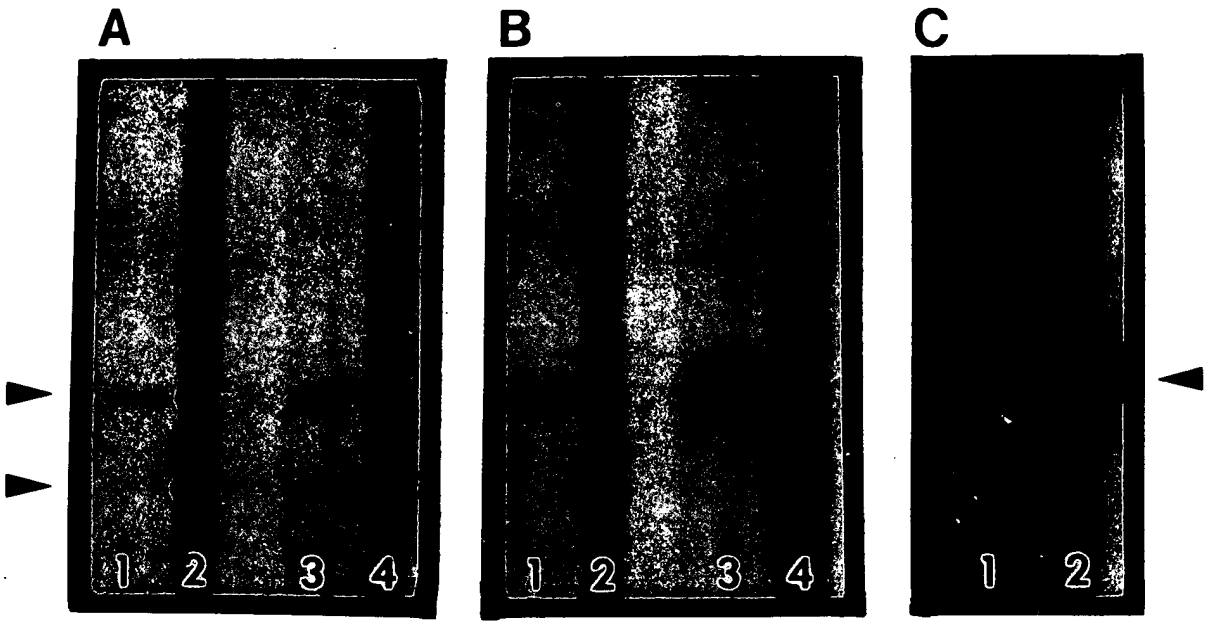

Figure 7. **ATP-dependent release and concomitant phosphorylation of grp 75 from immunoprecipitates.** HeLa cells growing at 37°C were labeled with [^{35}S]-methionine for 2 h, the label was removed, and the cells were further incubated for 1 h in the absence of label. The cells were lysed by addition of nonionic detergent and inclusion of apyrase to deplete ATP. Native immunoprecipitations were performed using antibodies to either hsp 58 or grp 75 as described previously. After washing the resultant immunoprecipitate with RIPA buffer, the immunoprecipitates were washed and resuspended in 20 mM Mes, pH 6.2, 0.1% Triton X-100, 10 mM $CaCl_2$ (kinase buffer). (A) To the hsp 58 immunoprecipitates was added either 10 μM of the ATP analogue AMPPNP or 1 μM ATP, and the samples were incubated for 30 min at 30°C. After the incubation the samples were centrifuged, the supernatant was removed, and the protein A-sepharose antibody-antigen complex was washed once with cold PBS. Laemmli sample buffer was added to the supernatant and to the pellet, and the samples were incubated at 100°C for 5 min. Equal percentages of the supernatant and pellet were then analyzed by SDS-PAGE and fluorography. Shown in lane 1 is the supernatant and in lane 2, the pellet of the hsp 58 immunoprecipitate incubated with 10 μM of the ATP analogue. Shown in lane 3 is the supernatant and in lane 4 the pellet of the hsp 58 immunoprecipitates incubated with 1 μM ATP. The positions of grp 75 and hsp 58 are indicated on the left. (B) An aliquot of the hsp 58 immunoprecipitates (described above) was incubated with either 10 μM of the ATP analogue AMPPNP or 1 μM ATP supplemented with 10 μCi of γ-[^{32}P]-ATP exactly as described above. After a 30-min/30°C incubation, the proteins released into the supernatant or those that remained bound to the protein A–sepharose were analyzed separately as described above. Shown is an autoradiogram of the gel. Shown in lane 1 is the supernatant and in lane 2 the pellet of the hsp 58 immunoprecipitates incubated with 10 μM of the ATP analogue plus γ-[^{32}P]-ATP. Shown in lane 3 is the supernatant and in lane 4 the pellet of the hsp 58 immunoprecipitates incubated with 1 μM ATP plus γ-[^{32}P]-ATP. (C) HeLa cells were solubilized in nonionic buffer, apyrase was added, and the lysates were incubated for 15 min at 4°C. Native immunoprecipitations were performed with the anti-grp 75 antibody, and the immunoprecipitate was washed extensively with RIPA buffer, then incubated in kinase buffer as described above. Unlabeled ATP (1 μM), supplemented with 10 μCi of γ-[^{32}P]-ATP, was added, and the sample was incubated for 30 min at 30°C. After the incubation the sample was centrifuged and the proteins present within the supernatant or the protein A–sepharose pellet were analyzed separately by SDS-PAGE. Shown is the autoradiogram of the gel. Shown in lane 1 is the supernatant and in lane 2 the pellet. The position of grp 75 is indicated on the right.

newly synthesized proteins, were no longer observed to co-precipitate with grp 75 in the pulse-labeled cells provided a subsequent 1-h chase period. In the case of hsp 58, it also exhibited interactions with a number of newly synthesized proteins. One of the proteins co-precipitating with hsp 58 again was the newly synthesized F_1ATPase β-subunit. Similar to the results obtained with grp 75, most of the newly synthesized proteins that co-precipitated with hsp 58 in the pulse-labeled cells were no longer apparent in the cells allowed a subsequent 1-h chase period. Hence, it appears that, as newly synthesized proteins enter into the mitochondria, they interact with grp 75 and hsp 58. Most of these interactions are both transient and sensitive to ATP.

A number of interesting and informative differences were observed when these same experiments were performed using cells labeled in the presence of the amino-acid analogue of proline, Azc. First, in the pulse-chase experiments, grp 75 again was observed to interact with a large number of the newly synthesized and analogue-containing proteins. Unlike the situation with the normal cells, however, these interactions did not appear transient. Instead, even after the 1-h chase period, most of these

analogue-containing proteins were still found to co-precipitate with grp 75. In the case of hsp 58, whereas a number of newly synthesized proteins co-precipitated with hsp 58 under normal conditions, significantly fewer co-precipitated when the pulse-labeling was performed in the presence of Azc.

On the basis of these results and a large body of published data regarding the hsp 70 family and GroEL-related stress proteins, we suspect that both grp 75 and hsp 58 are important in mediating the import into and subsequent assembly of proteins within the mitochondria. The possible role of hsp 58 and grp 75 in mediating these events is summarized in Figure 8. As mitochondrial proteins are being synthesized within the cytoplasm, they, like most other cellular proteins, become complexed with the cytosolic forms of hsp 70 (i.e., 72/73) (Beckmann *et al.*, 1990). Perhaps by virtue of their amino-terminal mitochondrial signal sequence and the presence of bound cytosolic hsp 70, these newly synthesized proteins destined for the mitochondria are maintained in an unfolded conformation (Randall *et al.*, 1990). Once at the mitochondrial membrane, these "unfolded" or "translocation competent" precursors begin their entry into the organelle accompanied by the ATP-dependent release of hsp 72/73. Support for the idea that the cytosolic hsp 70 proteins are involved in this step, along with additional cytosolic components, has been provided by other investigators (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Sheffield *et al.*, 1990). As the translocating and presumably unfolded polypeptide enters into the mitochondria, it rapidly becomes complexed with that form of hsp 70 within the organelle, namely, grp 75. Binding to grp 75 may provide for the stabilization of the translocating and unfolded polypeptide as it is

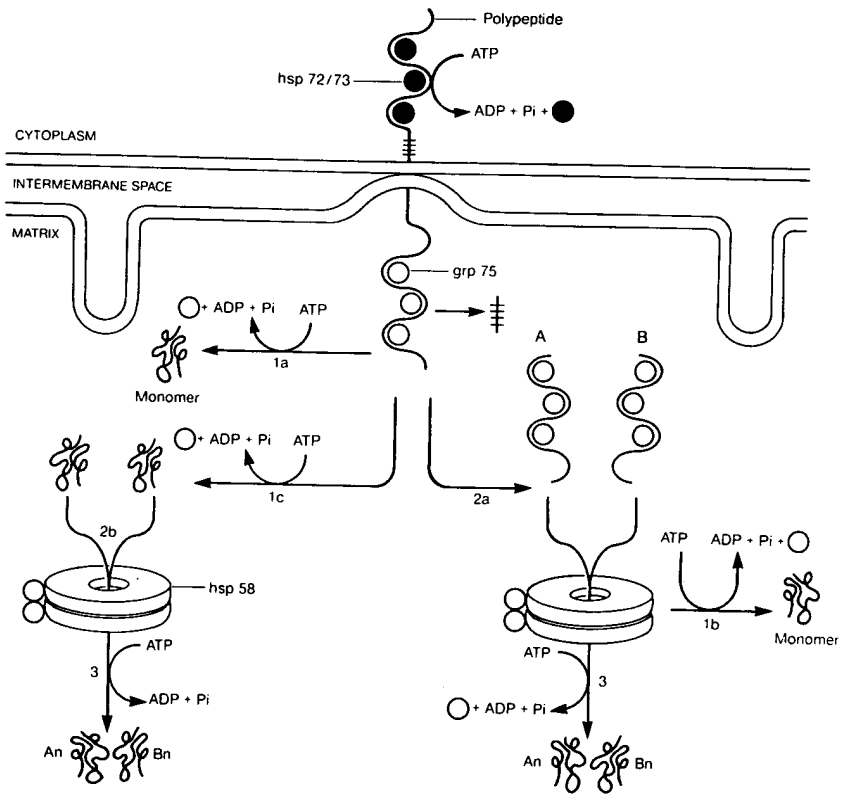

Figure 8. **A model describing the possible role of grp 75 and hsp 58 in mitochondrial protein import, folding, and assembly.** Newly synthesized proteins destined for the mitochondria are maintained in an unfolded or translocation competent state within the cytoplasm by virtue of their interaction with the cytosolic hsp 72/73. Translocation of the polypeptide into the mitochondria is accompanied by the ATP-dependent release of hsp 72/73. As the translocating and unfolded polypeptide enters into the mitochondria, it becomes complexed with grp 75, and the mitochondrial signal sequence is removed by signal peptidase. Once entirely inside the mitochondria, folding of the polypeptide commences, accompanied by the ATP-dependent release of grp 75 (1a). For some monomeric mitochondrial proteins, it remains possible that folding is also dependent on an interaction with hsp 58 (1b). For the assembly of oligomeric proteins, grp 75 is released from the monomer (1c); the monomer then moves to hsp 58 (2b) and is assembled into its oligomeric form (3). Alternatively, the monomer, still bound to grp 75, moves to hsp 58 (2a) and is assembled into its oligomeric form (3).

processed by the signal peptidase and may prevent the premature folding of the incoming polypeptide until its translocation into the organelle has been completed. Once entirely inside the mitochondria, the polypeptide begins to fold, accompanied by the ATP-dependent release of grp 75. For those mitochondrial proteins that are to be assembled into oligomeric structures, hsp 58 is called into play. Specifically, and as others have suggested, the large hsp 58 complex (a homooligomer of 14 subunits) serves as a scaffold by which monomeric proteins are assembled into their final oligomeric structure (Georgopoulous *et al.*, 1973; Sternberg, 1973; Rochan and Murialdo, 1983; Hemmingsen *et al.*, 1988; Ellis and Hemmingsen, 1989; Chang *et al.*, 1989). As is indicated in Figure 8, it is not clear whether grp 75 acts in concert with hsp 58 in the oligomerization process. Support for the two stress proteins working together was our observation that grp 75 could be co-precipitated with hsp 58 and that the complex was relatively stable. In addition, it remains possible that for some monomeric proteins both grp 75 and hsp 58 are required in the folding process.

How does this proposed model fit with the results presented here? First, a significant number of newly synthesized proteins were observed to co-precipitate with either grp 75 or hsp 58 in the pulse-labeled cells, most of which were no longer observed to co-precipitate after the subsequent chase period. Second, if the pulse-labeling was performed in the presence of the amino acid analogue Azc, the newly synthesized proteins again appeared to form a complex with grp 75. However, even after the subsequent 1-h chase period, most of the Azc containing radiolabeled proteins were still observed to co-precipitate with grp 75. Third, compared with the cells labeled under normal conditions, significantly fewer proteins synthesized in the presence of Azc were found to co-precipitate with hsp 58. It is these latter two points, along with previous studies of the hsp 70 and hsp 58 stress protein families, that leads us to suspect that proteins translocating into the mitochondria first interact with grp 75. Specifically, when the incoming newly synthesized mitochondrial protein contains the amino acid analogue, it binds to grp 75 but is not subsequently released, presumably owing to its inability to fold properly. Consequently, this "dead end" form of the incoming protein is presumably not released over to hsp 58 for its subsequent folding and/or higher ordered assembly. This scenario therefore could account for the fewer number of analogue-containing proteins co-precipitating with hsp 58 in the cells pulse-labeled in the presence of Azc.

Much of the proposed pathway appears consistent with previous studies of both the hsp 70 and GroEL (hsp 58) stress protein families and is analogous to a model that appeared after submission of our present study (Neupert *et al.*, 1990). For example, both the cytosolic hsp 70 proteins (hsp 72/73) and the form of hsp present within the lumen of the ER, grp 78 (BiP), have been reported to interact with other nascent polypeptides during the course of their maturation. Most pertinent here are the studies showing that BiP interacts with proteins as they enter into the ER lumen. BiP appears to stabilize the incoming protein as it is glycosylated and assembled into its final mature form. Moreover, loss of normal BiP function results in an eventual cessation of protein import into the ER (Vogel *et al.*, 1990). During the course of this work we learned that Kang *et al.* (1990) isolated a temperature-sensitive mutant of SSC1, the yeast equivalent of grp 75. At the nonpermissive temperature, blockage of protein import into mitochondria was observed. These investigators further demonstrated that the incoming protein became arrested within the mitochondrial translocation channel. These results indicate that, in the absence of functional BiP or grp 75, proteins entering into the appropriate organelle begin to fold prematurely and probably become arrested within the translocation apparatus. Consequently, a picture has emerged in which the various members of the hsp 70 family appear to function by binding to and stabilizing the nascent chains of unfolded polypeptides.

With respect to the GroEL proteins, numerous studies have demonstrated their role in higher ordered protein assembly. In the case of bacterial GroEL, the protein appears essential in facilitating the organization of lambda phage head protein monomers into their final oligomerized, prohead structure (Georgopoulos *et al.*, 1973; Sternberg, 1973; Rochan and Murialdo, 1983). In plants, a GroEL-related protein, the Rubisco binding protein, appears to catalyze the assembly of the large multimeric Rubisco complex. Finally in this regard are the studies of Chang *et al.* (1989), who reported that mutations in the yeast form of GroEL (termed hsp 60) result in the failure of multimeric enzymes to properly assemble within the mitochondria. Hence, these previous studies demonstrating a role for GroEL proteins in facilitating protein oligomerization events form part of the basis for our suggestion that it is hsp 58 (perhaps in conjunction with a portion of grp 75) that is impor-

tant for protein oligomerization events in the mitochondria.

Finally, our data may also reflect on the mechanism(s) by which hsp 70 proteins facilitate their release from the target polypeptide. We showed that grp 75 could be isolated in a complex with hsp 58 and that, in such hsp 58 immunoprecipitates, addition of ATP resulted in the release of grp 75. Moreover, accompanying its release was the apparent autophosphorylation of grp 75. This apparent autokinase activity of grp 75 is similar to what has been observed previously for the bacterial homologue dnaK, as well as to a recent study examining grp 75 itself (Zylicz *et al.*, 1983; Leustek *et al.*, 1989). Similar to these previous reports, we find that the autokinase activity of grp 75 is maximal at lower pH (~6.0) and is calcium dependent. These conditions appear most relevant, considering the fact that the environment of the mitochondria is maintained at a slightly lower pH and contains relatively higher levels of calcium than that found in the cytoplasm. Consequently, we are pursuing the possibility that the mechanism by which grp 75 is released from its target involves an autophosphorylation event that may result in a change in its own conformation and subsequent release from its substrate.

Materials and methods

Cell culture and metabolic labeling

All experiments employed HeLa cells growing on plastic dishes at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum (CS). For studies examining the relative half-lives of mitochondrial protein precursors and/or their conversion rate to the mature form of the protein, HeLa cells were incubated with 20 μM CCCP for 10 min (Reid *et al.*, 1982). While still in the presence of CCCP, the cells were labeled with [^{35}S]-methionine for 30 min at 37°C. The radiolabel was removed, and one group of cells was further incubated in the presence of CCCP for either 30 min or 2 h. Alternatively, to follow import of the protein precursors into the mitochondria, cells were treated with CCCP for 10 min and then labeled with [^{35}S]-methionine in the presence of CCCP for 30 min. The medium containing CCCP and radiolabel was removed and the cells were washed with medium containing 0.05% 2-mercaptoethanol, then further incubated in DMEM plus 5% CS for either 30 min or 2 h. After the appropriate incubation period, the cells were solubilized in Laemmli sample buffer and heated at 100°C, then denaturing immunoprecipitations were performed.

For experiments examining protein–protein interactions under steady-state conditions, HeLa cells were labeled for 2 h, the label was removed, and the cells were further incubated in the absence of radiolabel for 1 h. Cell lysates were prepared as described below. For some experiments, HeLa cells were incubated with 5 mM Azc for 4 h at 37°C. While still in the presence of the analogue, the cells were labeled for 2 h with [^{35}S]-methionine, then the label was removed, the cells were further incubated in the presence of Azc for 1 h, and cell lysates were prepared.

For pulse-chase experiments, HeLa cells, either incubated under normal conditions or preincubated with 5 mM Azc for 4 h, were pulse-labeled with [^{35}S]-methionine for 15 min in either the absence or presence of the analogue. Some of the cells were immediately harvested (below), whereas for others, the label was removed, and the cells were washed with fresh culture medium, further incubated in the absence or presence of Azc for 1 h at 37°C, and then harvested.

Preparation of cell lysates and immunoprecipitation analysis

For some experiments (e.g., Figure 1) whole cell lysates were used for the immunoprecipitation analysis. After labeling, the cells were washed with PBS and solubilized directly into RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS in PBS). For most other experiments, a crude mitochondrial preparation was used as the starting material for the immunoprecipitation. After the appropriate labeling period, the cells were washed with PBS and then incubated in cold hypotonic buffer (10 mM $NaPO_4$, pH 7.4) plus 10 U/ml apyrase (Sigma, St. Louis, MO) for 5 min on ice. The cells were scraped from the plates, adjusted to 1 mM $MgCl_2$, dounce homogenized, vortexed vigorously, and centrifuged at 13 000 × *g* for 20 min at 4°C. The resultant pellet, containing nuclei and mitochondria, was solubilized by the addition of 100 μl of PBS containing 1 mM $MgCl_2$ and 0.1% Triton X-100. Apyrase (final 10 U/ml) or ATP (final 2 mM) was added, and the samples were incubated at 4°C for 15 min. RIPA buffer (1 ml) was added, the material was clarified, and the supernatant was then used for the immunoprecipitation analysis.

Denaturing immunoprecipitations from cells solubilized in Laemmli sample buffer and heated at 100°C were performed as described previously (Mizzen *et al.*, 1989). For native immunoprecipitations, the cells or crude mitochondria were solubilized in RIPA buffer, and the amount of incorporated radioactivity was determined by TCA precipitation (using bovine serum albumin as carrier). Equal amounts of total radioactivity were used for each immunoprecipitation within a particular experiment (e.g., pulse and pulse-chase). The samples were diluted with RIPA buffer; protein A–sepharose was added; and the mixture was incubated on a rotary shaker for 30 min at 4°C, then clarified in an Eppendorf centrifuge. The supernatant was removed and to it was added the appropriate antibody. Antibodies used included rabbit anti-mouse IgG (control), rabbit anti-F_1ATPase α-subunit, rabbit anti-hsp 58, and rabbit anti-grp 75 (Mizzen *et al.*, 1989). After incubation with the antibody for 2 h at 4°C, the immune complexes were captured by the addition of protein A–sepharose and washed three to five times with RIPA buffer. Proteins present in the immunoprecipitate were solubilized by the addition of Laemmli sample buffer and heated at 100°C for 5 min.

For experiments analyzing the release of grp 75 from the immunoprecipitates, HeLa cells were labeled with [^{35}S]-methionine for 2 h at 37°C, the cells were harvested in RIPA buffer and treated with apyrase, and native immunoprecipitations were performed using either the hsp 58 or grp 75 antibodies as described above. After washing with RIPA buffer, we washed the immunoprecipitates twice with PBS and then once with 20 mM Mes, pH 6.2, 1 mM $MgCl_2$, and 0.1% Triton. After resuspending the protein A–sepharose antibody-antigen complex in this latter buffer, we adjusted the samples to 10 mM $CaCl_2$ and incubated them in the presence of 1 μM ATP or 10 μM of the ATP analogue AMPPNP for 1 h at 30°C. After the incubation, we centrifuged the samples at 13 000 × *g*, carefully removed the supernatant, adjusted it to 1× Laemmli sample buffer, and heated it at 100°C for 5 min. The pellet containing the im-

mune complex was washed once with PBS, and the proteins were solubilized by the addition of Laemmli sample buffer and heating at 100° for 5 min. Equal percentages of the supernatant and pellet were analyzed by SDS–PAGE.

To examine the possible autophosphorylation of grp 75 during its release from the hsp 58 immunoprecipitates, we incubated an aliquot of the [^{35}S]-methionine-labeled immunoprecipitates (described above) with 1 μM ATP or 10 μM of the ATP analogue (AMPPNP) plus 10 μCi of γ-[^{32}P]-ATP (Amersham, Arlington Heights, IL; 5000 Ci/mM) for 30 min at 30°C. After the incubation, we analyzed the supernatant and pellet separately as described above.

Examining possible grp 75 autokinase activity

Purified guinea pig liver mitochondria or purified grp 75 were examined for kinase activity. Mitochondria were purified essentially as described by Pederson *et al.* (1978), and grp 75 was purified from these mitochondria as described previously (Mizzen *et al.*, 1989). After solubilization of the mitochondria with Triton X-100 (final 0.2%), the mitochondria or purified grp 75 was incubated at either pH 6.2 (100 mM Mes) or pH 8.2 (100 mM Tris) in the presence or absence of calcium or magnesium (details provided in figure legend 6). To each reaction was added 1 μM of ATP supplemented with 10 μCi of γ-[^{32}P]-ATP (Amersham; 5000 Ci/mM), and the sample was incubated at 30°C for 60 min. The reactions were terminated by the addition of 5× Laemmli sample buffer heated at 100°C, and the [^{32}P]-radiolabeled proteins were analyzed by SDS-PAGE and autoradiography.

One- and two-dimensional gel electrophoresis

One-dimensional SDS-PAGE was performed using 12.5% gels prepared as described by Laemmli (1970). Two-dimensional gel electrophoresis, employing isoelectric focusing (80% pH 5–7 and 20% pH 3–10 ampholines) in the first dimension followed by SDS-PAGE in the second dimension, was done as described previously (Welch *et al.*, 1983).

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